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Demographics of Natural Oral Infection of Mosquitos by Venezuelan Equine Encephalitis Virus

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The within-host diversity of virus populations can be drastically limited during between-host transmission, with primary infection of hosts representing a major constraint to diversity maintenance. However, there is an extreme paucity of quantitative data on the demographic changes experienced by virus populations during primary infection. Here, the multiplicity of cellular infection (MOI) and population bottlenecks were quantified during primary mosquito infection by Venezuelan equine encephalitis virus, an arbovirus causing neurological disease in humans and equids.

RNA viral infections generally generate large and diverse populations within the infected host. This diversity plays a key role in important epidemiological and evolutionary processes (1–3). However, several steps during transmission can constrain the demographics and genetics of the virus population, with host primary infection being one of the main barriers.

During primary infection, the number of initially infected cells is not infinite, potentially lowering the size and genetic diversity of the colonizing population compared to that present in the donor host. The multiplicity of cellular infection (MOI) in those cells is thus a fundamental parameter determining the demographics and genetics of the colonizing population. The MOI is the number of genomes of a virus that enter and replicate in a cell (4). This parameter impacts the size of population bottlenecks during primary infection because, for a given number of primary infected cells, the higher the MOI, the larger the colonizing population. Furthermore, the MOI also influences genetic diversity, as it largely defines the intensity of genetic exchange and complementation among genotypes during cell coinfection.

Despite the importance of the MOI and population bottlenecks, there is a striking lack of formal estimates of these parameters, not only during primary infections but throughout the virus transmission cycle (reviewed in reference 4). Here, we use available data sets to estimate the demographics of Venezuelan equine encephalitis virus (VEEV), a mosquito-borne arbovirus, during the primary oral infection (i.e., the midgut infection) of its mosquito vectors.

VEEV is an alphavirus in the family *Togaviridae* that periodically causes epidemics and equine epizootics. It circulates in two transmission cycles, the epizootic/epidemic (here called epizootic) and enzootic cycles, with distinct strains and mosquito species associated with each cycle. Enzootic VEEV strains are generally associated with the absence of disease in equids, with the exception of certain subtype IE strains (5), and transmission cycles are associated with forest mosquitos, mainly *Culex* (*Melanoconion*) spp., and rodent hosts. Epizootic strains tend to be highly pathogenic to equids and can be transmitted by mosquito species that feed principally on large mammals, like *Aedes* (*Ochlerotatus*) *taeniorhynchus*. Vector susceptibilities vary widely, with enzootic VEEV strains typically exhibiting highly efficient but specific infectivity for sympatric, enzootic vectors, while epizootic strains show less-efficient infection but exploit a wider range of mosquito species.

Previous studies have assessed the extent of midgut infection in two VEEV strain/mosquito species pairs, each representative of either the epizootic or the enzootic cycle: the epizootic 3908 strain (subtype IC)/*A. taeniorhynchus* and the enzootic 68U201 strain (subtype IE)/*Culex taeniopus* (6, 7). The methods used (5) were similar for both pairs. Two types of virus-like particles containing replicons (i.e., defective genomes that undergo replication without generating infectious virus to spread) were generated. Each particle type contained a replicon expressing either green or cherry fluorescent protein (GFP or CFP, respectively). Mosquitos were exposed to a blood meal spiked with a mixture of the two particles. After 24 h, guts were dissected and cells showing replicon-derived fluorescence were counted.

Using these data (Table 1 and see Table S1 in the supplemental material), we estimated the MOI during primary infection in the two transmission cycles (Table 1). We used the method developed in Gutiérrez et al. (8) for the epizootic pair. Briefly, this method uses a maximum-likelihood approach and infers the MOI that maximizes the chances of observing the number of cells showing coinfection by the two replicons given the total number of infected cells and the frequencies of the GFP- and CFP-expressing replicons in the ingested blood meal. Since no coinfected cells were observed in the enzootic pair, we used an alternative approach.

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TABLE 1 MOI and bottleneck size during mosquito primary infection in the epizootic and enzootic VEEV pairs[a]

Pair	No. of midguts examined	No. of infected cells[b] (range)	No. of coinfected cells[b]	Mean GFP expression frequency	MOI[c]	Mean MOI in infected cells	N_e (95% CI)
Epizootic	13	159 (21–433)	30	0.626	0.80 (0.73–0.88)	1.45	83 (54–210)
Enzootic	5	1,762 (1,064–3,317)	0	0.497	(0–0.00136)	1	520 (313–2,553)

[a] The MOI is the number of genomes per cell, and the bottleneck size (N_e) is the number of genomes per midgut. 95% CI, 95% confidence interval.
[b] Mean number per gut.
[c] Maximum-likelihood estimate (for the epizootic pair only) and 95% confidence interval (in parentheses).

We estimated a 95% confidence interval for the MOI directly from the value above which the probability of observing no coinfected cell was less than 0.05, through numerically solving for λ in the following equation:

$$\prod_{i=1}^{m}\left(1-\frac{(1-e^{-\lambda f_i})(1-e^{-\lambda(1-f_i)})}{1-e^{-\lambda}}\right)^{n_i}=0.05$$

where λ is the MOI, f_i is the frequency of the GFP-expressing replicon in the blood meal of the i^{th} (among m) examined mosquito midguts, and n_i is the number of infected cells observed in the i^{th} midgut. The MOI differed by several orders of magnitude between the two virus-strain/mosquito-species pairs, as suggested by previous estimates of cell coinfection (6, 7). Figure 1 shows the inferred distribution of MOI values among the population of infected cells. Infection with an MOI above 1 was an extremely rare event in the enzootic pair (probability $< 10^{-3}$). However, in the epizootic pair with higher blood meal titers characteristic of viremic equines, 35% of the infected cells were infected by more than one genome, despite the fact that infected cells were 10 times less abundant (Table 1).

Direct comparison between the two pairs is difficult due to differences in the viral doses and vector susceptibilities. For example, differences in blood meal titers between the two pairs may have affected our estimates (inoculum doses were 6.5 $\log_{10}$ fluorescent units/ml and 8 $\log_{10}$ focus forming units/ml in the enzootic and epizootic pairs, respectively), as the viral load in the inoculum can influence the MOI *in vivo* (8, 9). Nevertheless, the absence of coinfected cells among the relatively large number of infected cells in the enzootic vector strongly suggests a superinfection exclusion phenomenon limiting the MOI (10), a situation in which important increases in the MOI with increasing oral doses are unlikely. Further analysis of the potentially different infection mechanisms between the two VEEV strains is limited by the refractoriness to infection of *C. taeniopus* by the epizootic strain. However, our estimates elicit testable predictions about the compositions of viral populations. For example, there might be differences in the frequencies of recombinant or defective genotypes between epizootic and enzootic populations.

We next explored the potential for within-cell interactions among viral genotypes during primary infection in the epizootic couple. Imagine a population of the epizootic strain composed of two genotypes: a wild-type genotype and a defective genotype. The defective genotype must coinfect cells with the wild-type to replicate via complementation of defective functions, a situation mimicking natural arbovirus populations (11) and potentially influencing epidemiology (12). Under these assumptions, the number of cells coinfected by both genotypes during primary infection can be estimated using three parameters: the frequency of the genotypes in the blood meal, the MOI, and the number of infected cells per gut. Figure 2 shows how the number of coinfected cells changes with the frequency of the defective genotype under the parameter values estimated for the epizootic pair. For example, this number reaches around 11 cells for a 10% frequency of the defective genotype. It would be interesting to characterize the genotype diversity of VEEV populations in vertebrate blood, in particular the frequency of defective genotypes, to estimate the probability of their maintenance during primary mosquito infection.

Using the same data sets, we also estimated the population bottleneck, N_e, endured by VEEV during blood meal ingestion and midgut infection. The methodology used is based on F_{st} statistics and uses genetic variance within and between populations (i.e., the virus populations in the inoculum and midguts) to estimate the effective population size (13). Bottleneck sizes ranged

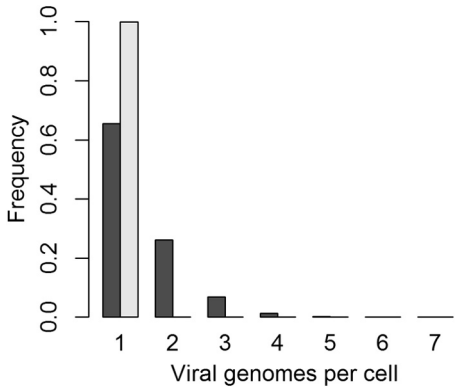

FIG 1 Distribution of the numbers of viral genomes that infect a cell in the epizootic (black bars) and enzootic (gray bars) VEEV pairs. In the epizootic pair, the frequencies of cells infected by 4, 5, 6, and 7 viral genomes are 0.013, 0.002, 2×10^{-4}, and 4×10^{-5}, respectively.

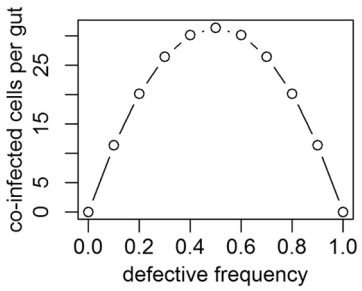

FIG 2 Estimation of cell coinfection by hypothetical wild-type and defective genotypes in relation to the frequency of the defective genotype in the ingested blood meal (parameter values are the mean values in the epizootic model [MOI = 0.80]; number of infected cells per midgut = 159).

from several tens in the enzootic model to several hundred genomes in the epizootic model (Table 1). The distribution of the estimates of the enzootic model overlapped a previous estimate obtained with the same model (previous estimate, 1,218 genomes ± 1,318 [mean ± standard deviation] [14]). Again, comparisons of values between the two VEEV pairs should be made cautiously due to differences in the experimental design potentially influencing bottleneck sizes. Severe bottlenecks, on the order of single digits, are the rule during primary infection in the few virus models analyzed so far, despite involving unrelated viruses and different transmission modes (15–20). Here, VEEV population sizes were between 1 and 2 orders of magnitude higher. Larger populations at primary infection may be crucial during the arboviral cycle of VEEV because they can preserve diversity and facilitate adaptation during the compulsory alternation between arthropod and vertebrate hosts (21). Future work could use the approach presented here to explore the conservation of the observed pattern during primary vertebrate infection by VEEV as well as in other arbovirus models.

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